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p19^{Arf} limits primary vitreous cell proliferation driven by PDGF-B

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Abstract

Arf encodes an important tumor suppressor, p19^{Arf}, which also plays a critical role to control hyperplasia in the primary vitreous during mouse eye development. In the absence of *Arf*, mice are born blind and display a phenotype closely mimicking severe forms of the human eye disease, persistent hyperplastic primary vitreous (PHPV). In this report, we characterize p19^{Arf} expression in perivascular cells that normally populate the primary vitreous and express the *Arf* promoter. Using a new *ex vivo* model, we show that these cells respond to exogenous Tgfβ, despite being isolated at a time when Tgfβ has already turned on the *Arf* promoter. Treatment of the cells with PDGF-B ligand doubles the population of cells in S-phase and ectopic expression of *Arf* blunts that effect. We show this effect is mediated through Pdgfrβ as expression of *Arf* represses expression of Pdgfrβ mRNA and protein to approximately 60%. p53 is not required for *Arf*-dependent blockade of PDGF-B driven proliferation and repression of Pdgfrβ protein as ectopic expression of *Arf* is still able to inhibit the 2-fold increase in the S-phase fraction of cells upon treatment with PDGF-B. Finally, induction of mature miR-34a, a microRNA previously identified to be regulated by p19^{Arf} does not depend on p53 while the expression of the primary transcript does require p53. These data corroborate that, as *in vivo*, p19^{Arf} functions to inhibit PDGF-B driven proliferation *ex vivo*.

Keywords

p19^{Arf}; Primary vitreous; PHPV; Pdgfrβ; Perivascular cells; p53; miR-34a

1. Introduction

During mammalian eye development, the hyaloid vascular system (HVS) in the primary vitreous provides nutrients to foster lens and retina development. This vasculature consists of the hyaloid artery, which feeds the vasa hyaloidea propria (VHP) and tunica vasculosa lentis (TVL) (Goldberg, 1997; Ito and Yoshioka, 1999). To achieve optimal vision, this fully formed vasculature regresses in later stages of eye development, resulting in the avascular and largely acellular secondary vitreous. Defects in HVS regression and primary vitreous maturation lead to ocular disease in children. This disease, variously known as either persistent hyperplastic primary vitreous (PHPV) or persistent fetal vasculature (PFV)

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(Goldberg, 1997; Haddad et al., 1978) covers a wide spectrum of severity from mere remnants of the hyaloid artery stalk to cellular hyperplasia leading to erosion of the lens capsule posteriorly and dysplasia and tractional detachment of the retina, leading to blindness (Goldberg, 1997; Haddad et al., 1978). Currently, therapeutic interventions largely hinge on surgical interventions to try to preserve or restore vision or remove a severely diseased eye (Hunt et al., 2005; Pollard, 1997).

The underlying pathogenetic mechanisms are likely to vary with disease severity, and this notion is supported in pre-clinical mouse models of the disease. These mechanisms can largely be divided into fundamental defects in pro-apoptotic processes needed to clear the cells from the vitreous and defects in processes that check hyperplastic expansion of primary vitreous cells – quite literally paralleling the two descriptors for the disease: PFV and PHPV. The former is reflected in mouse models with germ-line destruction of the p53 tumor suppressor gene (Ikeda et al., 1999) or angiopoietin-2 (Gale et al., 2002; Hackett et al., 2002) or in mice lacking macrophage-like hyalocytes (Lang and Bishop, 1993) or the WNT7B signals needed for them to exert cytotoxic effects on endothelial cells in the HVS (Lobov et al., 2005). In contrast, deregulated vascular endothelial growth factor (VEGF) (Mitchell et al., 1998; Rutland et al., 2007) or platelet-derived growth factor-B (PDGF-B) (Lei et al., 2010; Niklasson et al., 2010) support the hyperplastic expansion of primary vitreous cells and PHPV-like phenotype. Anti-proliferative factors have also been implicated. In particular, loss of the *Arf* tumor suppressor gene leads to hyperplasia of Pdgfrβ-expressing perivascular cells that normally flank the hyaloid artery mice mimics many aspects of PHPV, including microphthalmia; retrolental, fibrovascular mass that erodes the lens capsule and distorts the retina; and dense lens opacity resulting in blindness (Martin et al., 2004; McKeller et al., 2002). Although $Arf^{-/-}$ mice develop bilateral, severe eye disease, contrasting the typical clinical presentation, loss of *Arf* in just a subset of primary vitreous cells through somatic mosaic deletion leads to a more variable phenotype with incomplete disease penetrance – better mimicking the clinical scenario (Mary-Sinclair et al., 2014).

Because *Arf* is normally expressed in perivascular cells of the primary vitreous, it represents an essential component of normal mouse eye development (Martin et al., 2004; McKeller et al., 2002; Silva et al., 2005). *Arf* encodes $p19^{Arf}$, a nuclear protein best known for its capacity to negatively regulate Mdm2, thereby stabilizing (and activating) p53 (Honda and Yasuda, 1999; Weber et al., 1999). A linear pathway from $p19^{Arf}$ to p53 cannot fully account for the developmental effects, though, because p53 deficient mice usually have normal eyes, whereas *Arf* loss leads to PHPV in a variety of pure and mixed mouse strains (Ikeda et al., 1999; McKeller et al., 2002; Reichel et al., 1998). Clues to the biochemical effects of $p19^{Arf}$ first came from genetic studies showing that the hyperplastic phenotype is ameliorated in $Arf^{-/-}$, $Pdgfr\beta^{-/-}$ embryos (Widau et al., 2012), and $p19^{Arf}$ expression correlates with lower expression of Pdgfrβ in the developing eye, and in cultured mouse embryonic fibroblasts (MEFs) (Silva et al., 2005; Thornton et al., 2007; Widau et al., 2012). More detailed mechanistic studies – also conducted in MEFs and 10T1/2 fibroblasts – demonstrated that $p19^{Arf}$ expression can block *Pdgfrβ* mRNA transcription in a p53-dependent way and Pdgfrβ protein translation independently of p53 (Widau et al., 2012). Finally, miR34a, a microRNA known to be controlled by p53, is also induced by $p19^{Arf}$ and is required for p53-indendent

translational repression of Pdgfrβ (Iqbal et al., 2014a). Though this detailed molecular mechanism explains the ocular phenotype in the absence of *Arf*, it is important to recognize that the experiments were conducted in mouse fibroblasts, whereas the cells that normally express $p19^{Arf}$ are pericyte-like cells derived from the neural crest (Silva et al., 2005; Zheng et al., 2010). In this report, we functionally characterize and confirm aspects of *Arf* biology previously defined in other cell types and *in vivo* in an *ex vivo* cell culture model that represents the first model in which the *Arf* promoter is normally active.

2. Results

To verify that the aforementioned molecular and genetic pathway is relevant to the cells normally expressing *Arf*, we took advantage of primary cultures of primary vitreous cells (PVCs) that we purified by flow cytometry based on expression of a Gfp reporter in $Arf^{Gfp/Gfp}$ (functionally, $Arf^{-/-}$) animals (Iqbal et al., 2014b). We previously characterized the PVCs by global gene expression analysis that highlighted, among other things, their pericyte-like nature (Iqbal et al., 2014b). These cells are readily apparent as early as embryonic day (E) 12.5 (Martin et al., 2004; McKeller et al., 2002; Silva et al., 2005) and dramatically increase in number in the primary vitreous space by E13.5 (Silva et al., 2005) and Fig. 1A. Several pieces of data indicate that Tgfβ2 is required for *Arf* induction in the primary vitreous. First, $Tgfb2^{-/-}$ mice have a variety of developmental defects, including primary vitreous hyperplasia (Saika et al., 2001; Sanford et al., 1997), and this correlates with decreased expression of $p19^{Arf}$ (Freeman-Anderson et al., 2009). Second, transgenic expression of Tgfβ1 driven from the α-crystallin promoter can correct the primary vitreous hyperplasia in $Tgfb2^{-/-}$ mouse embryos, but not in $Arf^{-/-}$ embryos, which indicates that $p19^{Arf}$ is needed for the anti-proliferative effects of Tgfβ in the eye (Zheng et al., 2010). Dual immunofluorescence staining shows $p19^{Arf}$ and the Tgfβ receptor TbrII to be coexpressed, suggesting that this protein signals to induce *Arf* directly (Freeman-Anderson et al., 2009). This was confirmed to be true in MEFs and 10T1/2 cells: exogenous Tgfβ1, 2, or 3 increases Smad2/3 binding to the *Arf* gene, recruits RNA polymerase II, and then increases *Arf* mRNA and protein expression (Freeman-Anderson et al., 2009; Zheng et al., 2010). Interestingly, even though we isolated the PVCs by virtue of Tgfβ2-driven *Arf* promoter activation and Gfp expression, addition of Tgfβ1 (5 ng/ml) further increased *Gfp* mRNA expression in these cells *ex vivo* (Fig. 1B). Hence, the developmental signaling pathway that is critical for eye development is maintained in cultured PVCs. This finding is also consistent with our previous global gene expression analysis showing that components of the Tgfβ pathway defined as being important for *Arf* induction, such as *Smad2/3*, *Sp1* and *Cebpβ*, are expressed in PVCs (Iqbal et al., 2014b).

As highlighted above, $p19^{Arf}$ inhibits cell proliferation and, in the context of eye development, it specifically blunts mitogenic effects of PDGF-B by down-regulating the expression of Pdgfrβ (Silva et al., 2005). To test whether $p19^{Arf}$ similarly arrests cultured PVCs, we utilized propidium iodide staining followed by flow cytometry and quantification using the Watson Pragmatic Model Watson 1987 (Watson et al., 1987). We generated MSCV-based retroviral vectors containing *Arf* cDNA upstream of an IRES element driving expression of Rfp (Iqbal et al., 2014b). Transducing PVCs with the *Arf-IRES-RFP* vector significantly decreased the fraction of cells in S-phase with an accumulation of cells in G_1

and G_2 phases as compared to the Rfp control (Fig. 2A). Of note, it is not currently possible to study the effect of endogenous *Arf* expression in PVCs for several reasons. First, $p19^{Arf}$ expression from the wild type allele in an $Arf^{Gfp/+}$ mouse severely restricts cell accumulation *in vivo*, and would likely do so *ex vivo*. Indeed, earlier work with $Arf^{+/-}$ MEFs demonstrated that serial expansion (which would be required if attempting to propagate PVCs from the very small numbers in a phenotypically normal $Arf^{Gfp/+}$ eye) usually results in cells that have lost the remaining wild type allele (Zindy et al., 1998). Second, it is not yet possible to purify $Arf^{Gfp/+}$ PVCs before *Arf* is expressed as we depend on Gfp expression for the flow cytometry sorting, and the *Arf* promoter drives the Gfp reporter. Nonetheless, the capacity for ectopic expression of $p19^{Arf}$ to mimic the *in vivo* arrest provides a new opportunity for structure-function analyses of $p19^{Arf}$ and identification of downstream effectors in one of the very few cell types known to normally express this protein in the developing mouse.

As mentioned above, we know that primary vitreous hyperplasia in the $Arf^{-/-}$ mouse is driven by Pdgfrβ (Silva et al., 2005; Widau et al., 2012). Hence, we evaluated how PDGF-B, the ligand for this receptor, influenced PVC proliferation and the capacity for $p19^{Arf}$ to block the effects using the explanted cells. First, exposure of serum-starved PVCs to PDGF-B (50 ng/ml) for 16 h increased the S-phase fraction by over two fold, but ectopic expression of $p19^{Arf}$ completely abrogated this effect (Fig. 2B). We correlated this blockade with the ability for $p19^{Arf}$ to significantly repress *Pdgfrβ* mRNA and protein (Fig. 3A and B).We conclude that *ex vivo* studies of PVCs faithfully reflect the *in vivo* biology: PDGF-B drives PVC proliferation and *Arf* expression blocks it by a mechanism that leads to decreased Pdgfrβ expression.

Although $p19^{Arf}$ is most well-known for its capacity to sequester Mdm2 and thereby stabilize p53 (Honda and Yasuda, 1999; Weber et al., 1999), the protein also acts independently of p53 to inhibit ribosomal RNA processing (Sugimoto et al., 2003), associate with E2F1 and c-Myc to inhibit their trans-activating potential (Datta et al., 2004; Eymin et al., 2001; Qi et al., 2004) and promote sumoylation of Mdm2, nucleophosmin and other proteins (Rizos et al., 2005; Tago et al., 2005). Genetic evidence from mouse studies suggests that $p19^{Arf}$ likely controls primary vitreous expansion in a manner that does not strictly depend on p53. Ocular development is normal in most strains of mice lacking p53; however, certain pure BALB/c and pure C57Bl/6 lines of $p53^{-/-}$ mice have a PHPV/PFV-like phenotype with variable penetrance (Ikeda et al., 1999). Further, the developmental defect in C57Bl/6 mice is abrogated when the animals are bred into a mixed C57Bl/6 × 129/Sv background (Reichel et al., 1998). In contrast, PHPV consistently develops in $Arf^{-/-}$ mice in pure C57Bl/6 and pure 129/Sv lines as well as mixed C57Bl/6 × 129/Sv animals (McKeller et al., 2002).We have taken this to mean that while p53 may play a role in transcriptional repression of *Pdgfrβ* mRNA, *Arf* expression represses the protein through p53 independent mechanisms (Widau et al., 2012).

We utilized the PVC model to evaluate the role that p53 plays in *Arf*-dependent repression of Pdgfrβ mRNA and protein. Because our PVCs retain the p53 gene, we developed PVC sub-lines in which LMP-based retroviral vectors (obtained from S. Lowe, Memorial Sloan Kettering Cancer Center) delivered either control or p53-specific shRNA, and puromycin

was used to select pools of PVCs retaining p53 (shCTL) and those with p53 knockdown (shp53). Although not complete, the knockdown is functionally significant: ectopic expression of *Arf* in the shCTL cells robustly increases p53 (Fig. 5B, lanes 1 and 2), but similar transduction of *Arf* into shp53 cells only increases p53 to a level that is still lower than baseline in the control cells (Fig. 5B, compare lanes 2 and 4). As another functional measure, doxorubicin (Dox) augments the expression of p21Cip1, a well-known p53 target (Harper et al., 1993), in a dose-dependent manner; this effect is also dramatically decreased in the p53 knockdown PVCs (Fig. 4A). We also observed that the baseline S phase fraction was slightly increased in the shp53 PVCs at baseline (Fig. 4B, lanes 1 vs 5), but in both cases exogenous PDGF-B drove additional cells into S phase (Fig. 4B, lanes 1 vs 2 and 5 vs 6). Importantly, ectopic *Arf* expression blunted the mitogenic effects of PDGF-B in the presence and in the absence of p53 knockdown (Fig. 4B, lanes 2 vs 4 and 6 vs 8, and Fig. 4C).

We used this system to address the importance of p53 in two Arf-dependent responses: Pdgfrβ mRNA and protein repression and miR34a induction. First, as with the parental PVCs (Fig. 3A), ectopic *Arf* expression significantly decreased *Pdgfrβ* mRNA but this effect was completely negated when p53 was knocked down (Fig. 5A). In contrast, *Arf* expression still retained the capacity to repress Pdgfrβ protein in shp53 PVCs, though the effect was somewhat moderated (Fig. 5B). In a similar way, *Arf* can increase the expression of primary miR34a, but only in the presence of p53 (Fig. 5C), but p53 is at least partly dispensable for induction of mature miR34a – indicating a p53-independent role for $p19^{Arf}$ in processing of this microRNA (Fig. 5D). All of these molecular effects are similar to those that we observed *in vivo* during primary vitreous development and maturation (Martin et al., 2004; Silva et al., 2005; Widau et al., 2012).

3. Discussion

In the mouse, the mature testis, developing eye, and umbilical arteries are the only sites where numerous *Arf* expressing cells are normally found (Freeman-Anderson et al., 2009; Silva et al., 2005; Zindy et al., 2003). *Arf* expressing cells in the eye include those perivascular cells embracing the hyaloid artery and also cells scattered in the cornea (Silva et al., 2005; Thornton et al., 2007; Zindy et al., 2003). Primary vitreous hyperplasia is the only recognized developmental defect due to *Arf* gene inactivation (Martin et al., 2004; McKeller et al., 2002), and this ocular disease is due to Pdgfrβ-driven proliferation in the aforementioned perivascular cells (Silva et al., 2005; Widau et al., 2012). Because the PVCs are unique as the only cell type known to be altered without *Arf*, it was important to validate the functional and biochemical effects of $p19^{Arf}$ in these cells. Indeed, using this new *ex vivo* model, we have established the following: 1) The *Arf* promoter can be engaged by Tgfβ to induce *Arf* expression in PVCs *ex vivo*; 2) Ectopic *Arf* expression arrests PVC proliferation, including proliferation driven by PDGF-B; 3) Cell proliferation arrest by $p19^{Arf}$ correlates with repression of Pdgfrβ; 4) and *Arf* expression in PVCs checks proliferation, decreases Pdgfrβ protein, and induces mature miR-34a independently of p53. All of these new findings accurately reflect the *in vivo* effects of $p19^{Arf}$ during eye development.

Since the *Arf* cDNA was first cloned, most of the cell-based functional studies have been carried out in MEFs, a wide range of fibroblast cell lines, and cancer cell lines – none of which reflect a "normal" cellular context. Despite that inherent limitation, much has been gleaned from such studies. Indeed, our own laboratory has defined most of the aforementioned molecular and cell biological effects of $p19^{Arf}$ in MEFs and 10T1/2 fibroblasts (Silva et al., 2005; Weber et al., 2000). However, a more granular understanding of *Arf* biology is likely to depend on context – expressing the protein in the right cell type. Gaining a better understanding of the nature of the perivascular cells and how *Arf* influences their biology represents one such area. These particular PVCs are nearly unique for two reasons. First, they embrace blood vessels that undergo dramatic regression in the immediate postnatal period. Second, they are the only cells in the eye that express $p19^{Arf}$. We state they are "nearly" unique because the umbilical arteries branching from the internal iliac vessels represent another vascular system that becomes superfluous in the immediate postnatal period, and this is the only other vasculature enveloped by *Arf* expressing perivascular cells (Freeman-Anderson et al., 2009). Of note, we have not yet uncovered a defect in the umbilical vessels when $p19^{Arf}$ is absent. Nearly all work on *Arf* in the eye has focused on how it controls the number of these PVCs – essentially, how it blocks primary vitreous hyperplasia. Yet, the fact that *Arf* is expressed in the cells from E11.5, just as the hyaloid vessels are forming (Silva et al., 2005), and the expression is extinguished at P5, just as the vessels begin to regress (Mitchell et al., 1998), suggests another role for $p19^{Arf}$: it might actually be required to temporarily stabilize the otherwise transient vessels. Perivascular cells, especially pericytes that represent a particular type of vascular mural cell, provide trophic signals to underlying endothelial cells (Hanahan and Folkman, 1996; Yancopoulos et al., 2000). Having developed an *ex vivo* culture model for those PVCs, we are only now in a position to establish more sophisticated systems to study these potential heterotypic interactions with endothelial cells and the role that $p19^{Arf}$ may play.

Similar comments can be made about the emerging role that $p19^{Arf}$ can play as a microRNA regulator. We previously showed that this protein can increase the expression of miR-34a independently of p53 pathway, and miR34a is needed for post-transcriptional Pdgfrβ repression and p53-independent cell proliferation control (Iqbal et al., 2014a). Others have shown – using MEFs – that $p19^{Arf}$ physically interacts with Drosha, and this interaction both increases and decreases the expression of a wide range of microRNAs (Kuchenreuther and Weber, 2014). We do not yet understand the mechanism by which $p19^{Arf}$ can induce miR-34a independently of p53, nor do we know the full spectrum of microRNA changes that accompany *Arf* expression in PVCs that normally express the protein. Because both of these are likely to be cell context-dependent, conducting the work in PVCs may provide the correct perspective for such studies. Ultimately, these types of analyses may inform our understanding of PHPV/PFV pathogenesis.

4. Methods

4.1. Cell isolation and culture

PVCs were isolated from early post-natal $Arf^{Gfp/Gfp}$ mice. Method for isolation is described in detail in Iqbal et al. (2014b). PVCs were cultured in Pericyte Medium (PM) (ScienCell)

and passaged using trypsin/EDTA. Tgfβ1 (R&D Systems), was added to cell culture medium at a dose of 5 ng/ml; an equivalent volume of vehicle (4 mM HCl) was added into the medium as a control. PVCs were transduced with *MSCV-RFP* or *MSCV-Arf-RFP* retrovirus. 16 h post transduction, culture media was replaced. 50 ng/mL PDGF-B was added to the cells for 16 h prior to harvesting for cell cycle analysis.

4.2. RNA expression

Total RNA was extracted from PVCs with miRNeasy mini kit (Qiagen). For qRT-PCR, 1 μg of total RNA was reverse transcribed using Superscript III RT kit (Invitrogen) according to the manufacturer's recommendations. Quantitative RT-PCR (qRT-PCR) was performed with KAPA SYBR Green Mastermix (KAPA) on BioRad (CFX96). The PCR program consisted of 20 s at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 20 s. Primer quality was analyzed by dissociation curves. The expression of *Pdgfrβ* and *primiR34a* was normalized to *Gapdh*. *miR-34a* was normalized to *U6*.

4.3. Cell cycle analysis

Propidium Iodide (PI) (Sigma) staining was performed after cells were harvested by trypsin-EDTA and fixed in 70% ethanol. Fixed cells were washed in PBS and centrifuged at 1200 rpm for 5 min. Cells were resuspended in 0.3 ml PBS and RNaseA (Sigma) was added to the suspension to final concentration of 0.5 mg/ml. After 1 h of incubation at 37 °C, PI was added to the suspension to a final concentration of 10 μg/ml. PI-stained cells were analyzed for DNA content with a BD Calibur flow cytometer and Watson Pragmatic Model to calculate the distribution of cells in G_1, S, and G_2 cell cycle phases. Results are averaged from 3 biological replicates.

4.4. Western blot

Protein expression was examined by Western-blotting according to a standard procedure. The following antibodies were used: anti-$p19^{Arf}$ (Ab80, Abcam, 1:1000), anti-Pdgfrβ (AF1042, R&D, 1:1000), anti-Hsc70 (Sc-1059, Santa Cruz, 1:5000), anti-p53 (Sc-6243, Santa Cruz, 1:1000). Band intensity was quantified using the Odyssey Image Studio Lite system and normalized to Hsc-70. Results are averaged from 3 biological replicates.

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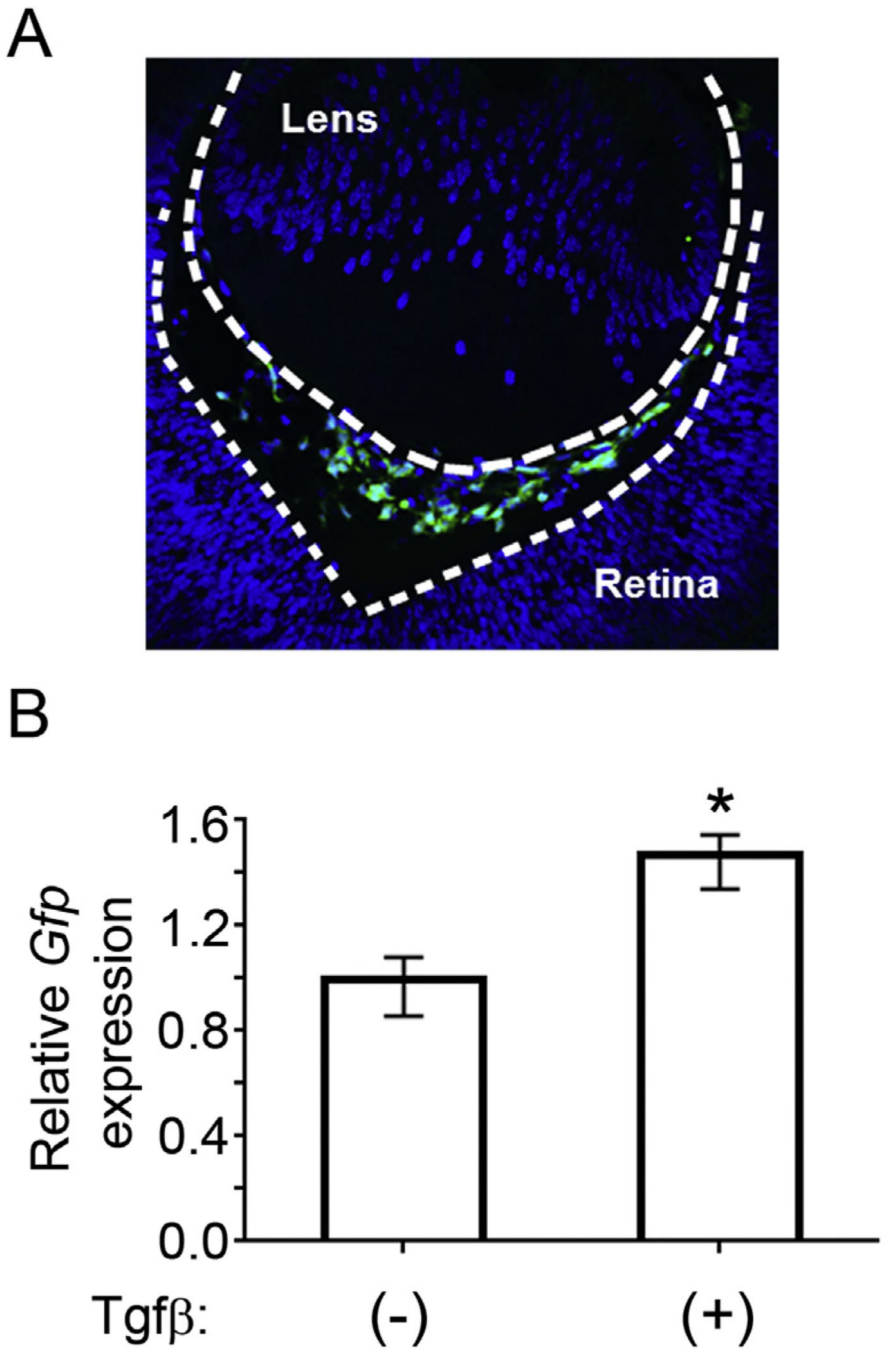

Fig. 1.

p19^{Arf} is expressed in the primary vitreous and responds to exogenous Tgfβ. A) Representative photomicrograph image of developing vitreous at E13.5 from *Arf*$^{Gfp/Gfp}$ mouse. Gfp expression, as a marker for *Arf* promoter activation, is robustly detected in cells of the primary vitreous space. B) Relative expression of *Gfp* as a surrogate marker for exon1β measured by qRT-PCR. PVCs were treated with 5 ng/mL Tgfβ1 or 4 mM HCl vehicle control. RNA was extracted 48 h after treatment. *Gfp* expression is normalized to *Gapdh* control (* = $p < 0.02$).

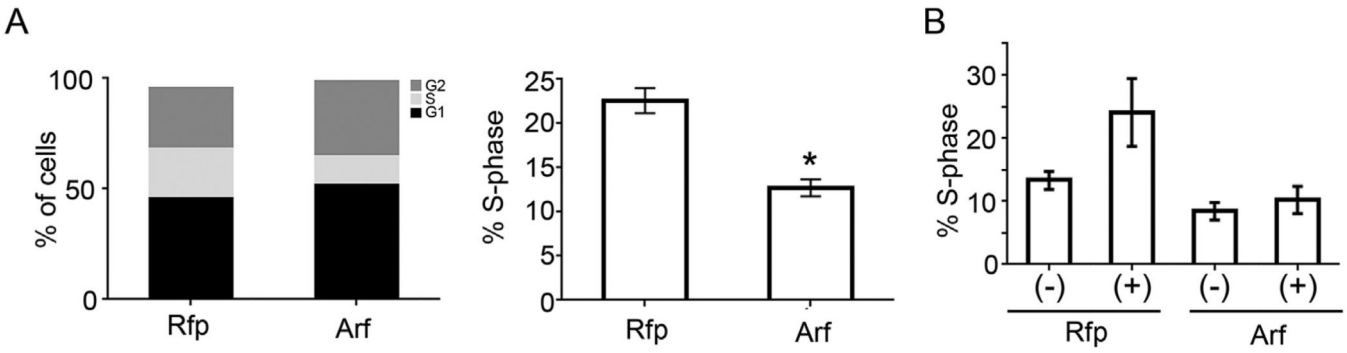

Fig. 2.

p19^{Arf} inhibits PDGF-B driven proliferation in the PVCs. A) Quantification of DNA content of PVCs as detected by propidium iodide and flow cytometry. PVCs were transduced with *MSCV-Rfp* control or *MSCV-Arf-Rfp* retrovirus and harvested 48 h later. Cell cycle phases were defined using the Watson Pragmatic Model. Quantification of S-phase fraction is shown on right. B) PVCs were transduced with *MSCV-Rfp* control or *MSCV-Arf-Rfp*. Cells were stimulated with 50 ng/mL PDGF-B for 16 h and cell cycle was analyzed as above (* = $p < 0.02$).

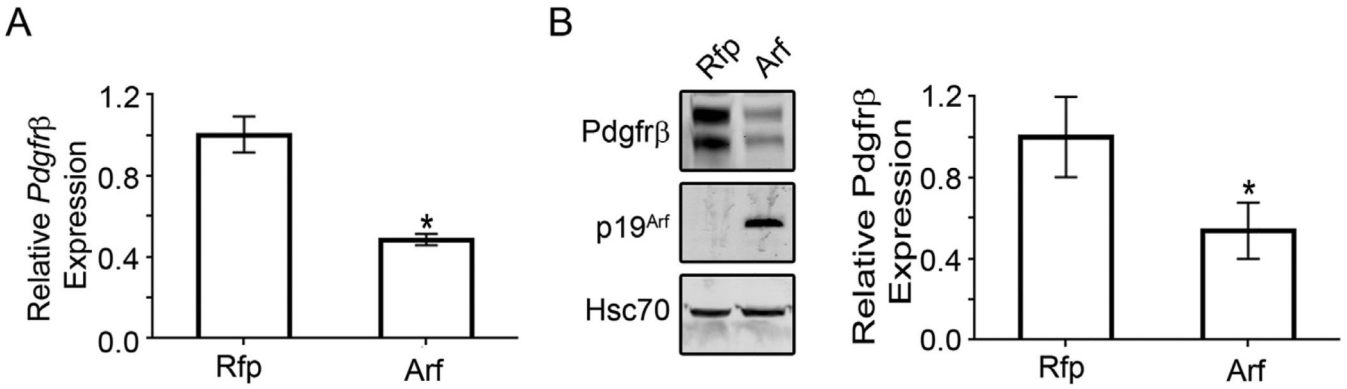

Fig. 3.

Pdgfrβ expression in PVCs depends on $p19^{Arf}$. A) Relative expression of *Pdgfrβ* mRNA as measured by qRT-PCR upon transduction with *MSCV-Rfp* control or *MSCV-Arf-Rfp*. B) Representative western blot showing Pdgfrβ, $p19^{Arf}$ and HSC-70 protein expression in lysates prepared from PVCs transduced with *MSCV-Arf-Rfp* retrovirus or *MSCV-Rfp* control. Western signal (on right) is quantified using the Odyssey Image Studio Lite system and normalized to Hsc-70 (* = $p < 0.05$).

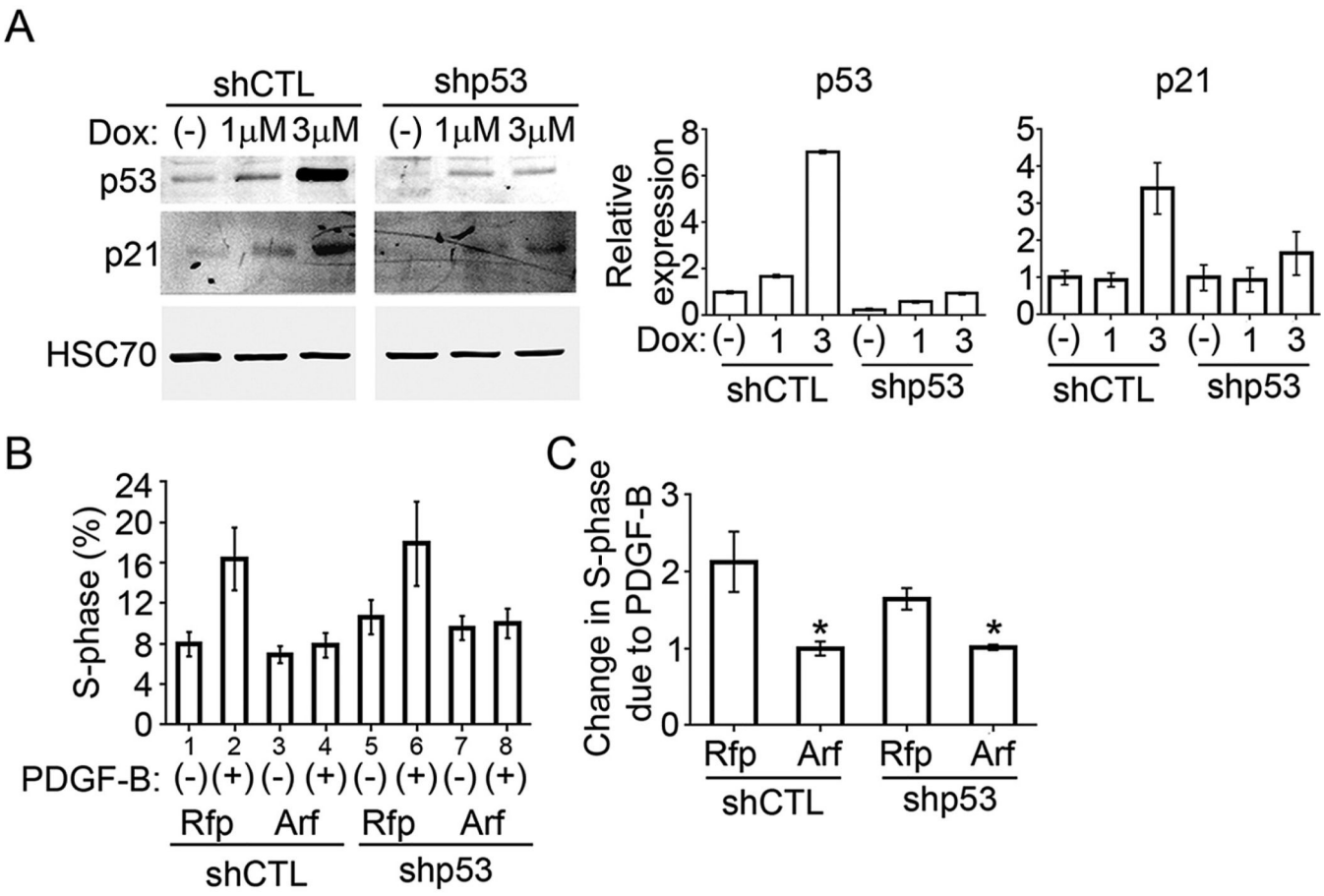

Fig. 4.

p19^{Arf} inhibits PDGF-B driven proliferation independently of p53. A) Representative western blot showing p53 induction in response to doxorubicin (Dox) treatment. p53 and p21 are induced upon treatment in shCTL cells but not shp53 cells. Quantification, normalized to Hsc-70, is on right. B) Quantification of percent of cells in S-phase as measured by propidium iodide and flow cytometry. shCTL or shp53 PVCs were transduced with *MSCV-Rfp* control or *MSCV-Arf-Rfp* and stimulated with 50 ng/mL PDGF-B for 16 h. C) Fold change of S-phase fraction of cells in response to PDGF-B (* = $p < 0.05$).

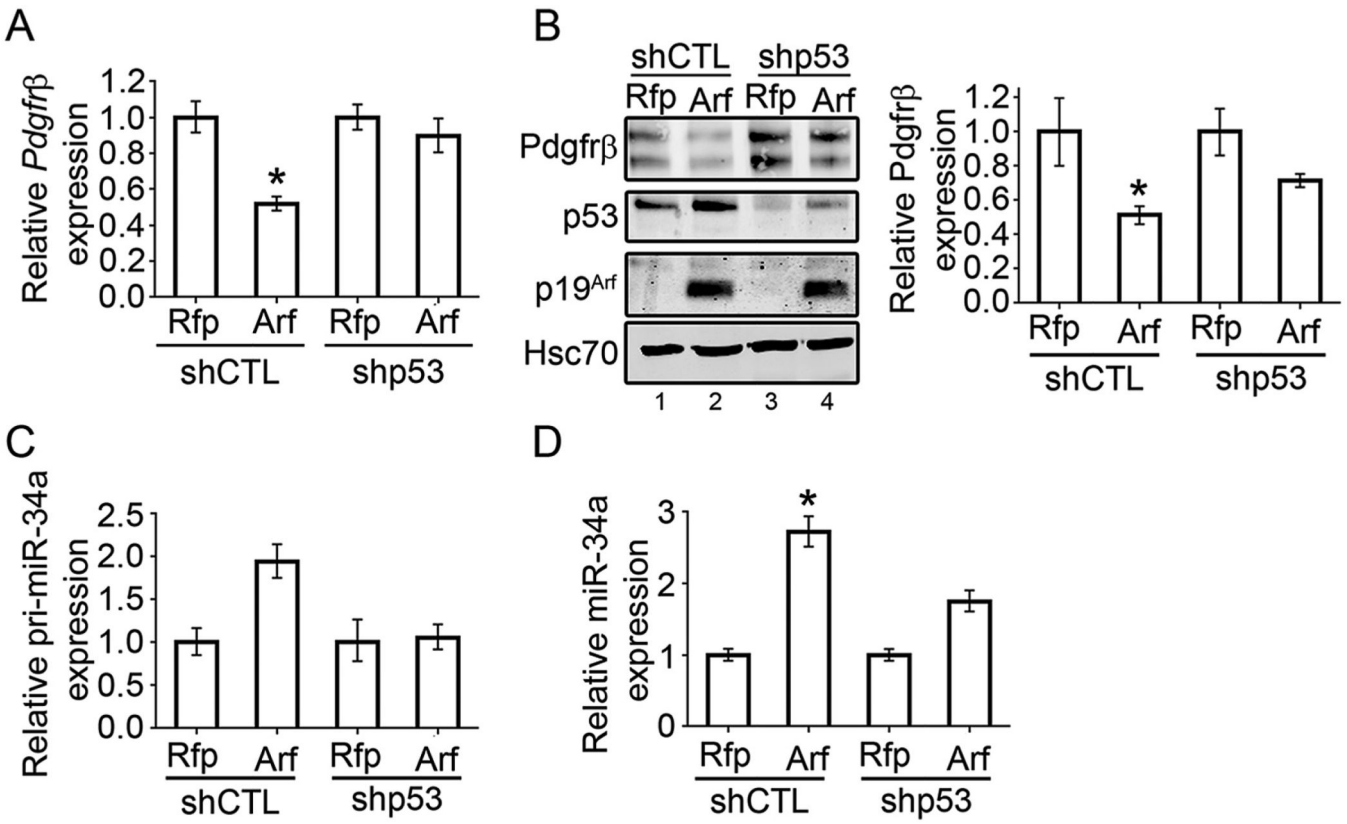

Fig. 5.

p53-dependent effects of $p19^{Arf}$ on Pdgfrβ and miR34a. A) qRT-PCR showing relative expression of *Pdgfrβ* mRNA upon transduction with *MSCV-Rfp* control or *MSCV-Arf-Rfp* retrovirus in shCTL or shp53 cells. B) Representative western blot showing Pdgfrβ, p53, $p19^{Arf}$ and Hsc-70 protein expression in lysates prepared from shCTL or shp53 cells transduced with *MSCV-Rfp* control or *MSCV-Arf-Rfp* retrovirus. Western signal (on right) is quantified using the Odyssey Image Studio Lite system and normalized to Hsc-70. C) Relative expression of *primary-miR34a* transcript in response to ectopic $p19^{Arf}$ expression in shCTL and shp53 cells. D) Relative expression of mature *miR34a* in response to ectopic $p19^{Arf}$ expression in shCTL and shp53 cells. *miR34a* is normalized to *U6* (* = $p < 0.05$).